Duchenne muscular dystrophy (DMD) is a lethal muscle wasting disease affecting approximately one in 3500 boys.\textsuperscript{1,2} Mutations in the dystrophin gene result in the loss of this protein from the sarcolemma of muscle fibers.\textsuperscript{1,2} Dystrophin deficiency does not consistently produce muscle degeneration at all life stages, in all muscle phenotypes, or in all animal models.\textsuperscript{3} In dystrophin-deficient skeletal muscle, mechanisms such as the immune/inflammatory response to injury appear to contribute substantially to muscle pathophysiology. Observations of activated immune cell infiltrates in dystrophic muscle suggest that the immune/inflammatory response may play a role in exacerbating the disease.\textsuperscript{1–7} Recently, individual macrophage subpopulations have been reported to influence muscle degeneration and regeneration depending on the proportion of these cells present. M1 macrophages are cytotoxic and pro-inflammatory while M2 macrophages promote muscle regeneration.\textsuperscript{8} Therefore, a shift in macrophage phenotype may be one mechanism that can regulate the dystrophic disease time course.\textsuperscript{8}

Complementary and alternative medicine (CAM) approaches, including the use of botanicals, are being pursued in the amelioration of DMD. Green tea is a widely consumed beverage believed to elicit anti-oxidant and anti-inflammatory properties.\textsuperscript{9,10} Green tea extract (GTE) is the hot water-soluble portion of unfermented \textit{Camellia sinensis} leaves, which contains high levels of polyphenols. The polyphenols in GTE are mainly composed of the catechins: galloカテchin (GC), epigalocatechin (EGC), epicatechin (EC), and epigallocatechin gallate (EGCG).\textsuperscript{9,11} These catechins are strong anti-oxidants that can quench reactive oxygen species (ROS) such as super oxide radical, singlet oxygen, hydroxyl radical, peroxyl radical, nitric oxide, nitrogen dioxide, and peroxynitrite.\textsuperscript{11} In GTE, ECGC is the most abundant polyphenol, accounting for 30–50% of total polyphenols, and is believed to provide the majority of the beneficial effects observed with green tea consumption.\textsuperscript{12} ECGC...
may lead to decreased inflammation through its anti-oxidant properties or through other mechanisms. EGCg has been shown to have effects on several signaling pathways including blockade of NF-κB activation by inhibiting IkB kinase (IKK) activity.10,13–18

The anti-oxidant potential of GTE may be beneficial in treating dystrophic muscle, because oxidative stress is believed to contribute substantially to muscle pathology.19,20 Evidence suggests that oxidative stress is involved in early disease stages and occurs before disease onset in mdx mice.21 In one study, diets supplemented with GTE (0.01% or 0.05%) were provided to mdx breeder pairs and their pups prior to and following weaning. At age 28 days, extensor digitorum longus (EDL) muscles of GTE treated mdx pups had significant reductions in areas of necrosis and regeneration.22 In a separate study, mdx mice treated with either GTE (0.05% or 0.25%) or ECGG (0.1%) for one to five weeks after weaning had increased anti-oxidant capacity, improved contractile properties, and decreased muscle pathology.14 These studies indicate the GTE and EGCg may provide beneficial CAM modalities for reducing oxidative stress and decreasing dystrophic muscle pathology during early disease stages, however; the role of these polyphenols in altering muscle pathology and inflammation has not been fully characterized. This study represents a detailed time-course characterization of pathological and inflammatory markers at which pre-natal and early GTE treatment of mdx mice decreases muscle wasting.

2. Materials and methods

2.1. Mice

C57BL/6j and mdx mice were obtained from our colony. Breeding mice and offspring were maintained in a supervised laboratory animal facility in polypropylene shoebox cages. Mice were given free access to public tap water via an automatic watering system, and fed a standard pelleted diet ad libitum. Decaffeinated GTE (90DCF; Sunphenon) (polyphenols > 80%, catechins > 80%, (α)-epigallocatechin-3-gallate (EGCG) > 45%, caffeine < 1%), was a kind gift from Taiyo International (Minneapolis, MN). Mdx breeder pairs were provided standard breeding pelleted diets (7004; Harlan Teklad) with no GTE, 0.25% GTE, or 0.5% GTE (0.01% or 0.05%) were weaned at age 21 days and then supplied with a standard maintenance diet (2018; Harlan Teklad) containing the same percentage of GTE provided to the breeder pair from which they came. C57BL/6j breeders were fed the standard breeding diet and weaned pups (n = 3–4 for each age group) were fed the maintenance control diet without GTE. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University and met or exceeded requirements of the Public Health Service/National Institutes of Health and the Animal Welfare Act.

2.2. Tissue collection

Mice of each genotype and treatment were selected at random from available cages. For tissue collection and for humane euthanasia, mice were sacrificed by carbon dioxide narcosis followed by secondary thoracotomy. Blood was collected via cardiac puncture. Tibialis anterior (TA) muscles were removed and were either flash frozen in liquid nitrogen and stored at −80 °C or fixed in 10% neutral buffered formalin until prepared for sectioning.

2.3. Serum analysis

Blood collected from mice was placed directly into Microtainer serum separator tubes (Becton Dickinson). Whole blood samples were allowed to clot for 30 min at room temperature. The tubes were then centrifuged for 2 min at 10,000g to separate serum. Serum creatine kinase (CK) assays were performed in the Clinical Pathology Laboratory at Virginia Tech, using an Olympus AU400 chemistry analyzer (Olympus America, Center Valley, PA).

2.4. Histopathological analysis

Formalin fixed TA muscles were prepared for light microscopy by dehydrating using increasing concentrations of ethanol and xylene as transitional solvents. Tissues were infiltrated with paraffin polymer, sectioned at 3 μm, and stained with hematoxylin and eosin (H&E) on automated staining equipment. A quantitative analysis of muscle histopathology was performed as follows. Degenerating fibers were identified as swollen eosinophilic, hyalinated, and pyknotic muscle fibers. Regenerating muscle fibers were identified by small diameter, centralization nuclei and basophilic cytoplasm. Necrotic fibers were identified as swollen muscle fibers with disrupted cell membranes invaded by inflammatory cells. Image analysis software (Image-Pro Plus, Media Cybernetics, Inc., Silver Spring, MD) was used to analyze tissue sections for pathological markers in fields selected with battlement technique from the entire cross section of the TA muscle. A grid was superimposed over each selected field and the number of intersections that overlay pathological markers was reported as a percent of the total intersections that overlay muscle tissue.

Frozen serial sections 10 μm thick were transferred to positively charged slides for immunohistochemical staining. Sections were fixed in 2% formalin for five minutes and rinsed in PBS. Macrosphages were identified by staining for anti-F4/80 (1:500; Serotec). After fixation, sections to be stained for NF-κB were permeabilized in 1% Triton X-100, rinsed in PBS, and covered with anti-NF-κB p65 specific for phosphorylated Serine 536 (1:100; Abcam). Sections were incubated with primary antibody overnight at 4 °C, rinsed in PBS, and immunodetection was performed using an anti-rat or anti-rabbit IgG labeled with horseradish peroxidase–diaminobenzidine system (R&D Systems). Additional sections were also incubated without primary antibody to ensure the presence of minimal background staining. The sections were then mounted and cover slipped for evaluation. The presence of macrophages and NF-κB staining was quantified using image analysis software (Image-Pro Plus, Media Cybernetics, Inc., Silver Spring, MD).

2.5. Statistics

Data were analyzed in a completely randomized design. Differences in muscle histopathology and serum CK were analyzed to determine the significance of the main effects and interactions. For the histopathology time course, the model was analyzed as a 2 × 5 factorial arrangement for genotype (C57BL/6j, mdx) versus age (14, 21, 28, 35 and 42 days). For histopathology and serum CK in GTE treatment studies, the model was analyzed as a 2 × 3 factorial arrangement for age (28 and 42 days) versus treatment (0%, 0.25%, 0.5% GTE). To determine the significance of the model, two-way analysis of variance (ANOVA) was performed using the general linear model. When the model was significant, the analysis was followed by Fisher’s Least Significant Difference multiple comparisons method (JMP 6.0.2 software. SAS Institute Inc., Cary, NC). Predetermined comparisons between GTE treated mdx mice and control mdx mice were made using Dunnett’s multiple comparisons method. Differences in macrophage
infiltration and NF-kB staining between GTE treatment groups were analyzed with one-way ANOVA. When significant differences were detected, Tukey’s Honestly Significant Difference post hoc test was used to determine differences between means. Differences for all analyses were considered significant at $P \leq 0.05$ and data were presented as the mean ± standard error.

3. Results

3.1. Microscopic muscle lesions in mdx mice

Muscle histopathology was quantified for mdx mice during the initial disease onset stages to further characterize and determine time points that represent early disease progression (Fig. 1). Systematically selected fields from TA muscles for both C57BL/6J and mdx mice were quantified (Fig. 2). Mdx TA muscles had normal fiber morphology at age 14 days, but by age 21 days the percent area of analyzed fields with normal fiber morphology was significantly decreased compared to C57BL/6J and was further decreased at age 42 days ($P \leq 0.05$). Degenerating muscle fibers peaked at age 21 days and represented 3.5% of the mdx TA area, but declined to lower levels thereafter. Immune cell infiltration of the muscle tissue and necrotic fibers were seen at age 21 days and peaked at age 28 days. At age 28 days immune cell infiltration and necrotic fibers accounted for 3.6% and 1% of abnormal morphology, respectively. Regenerating muscle fibers were the most abundant histopathological feature accounting for 28% to 61% of the TA area between ages 28 and 42 days ($P \leq 0.05$). Ages 28 and 42 days were selected as the primary time points for determining the effects of GTE on muscle pathology and inflammation because they represent critical time points in the disease process of the mdx mouse.

3.2. GTE treatments in mdx mice

Body mass was recorded for treated mdx mice and compared to control mdx mice to determine how GTE supplementation affected overall health. GTE supplementation did not result in a significant change in body mass for either treatment when compared to untreated mdx mice at either age (Average body mass in grams: 28 day: mdx = 13.4, mdx 0.25% GTE = 15.9, mdx 0.5% GTE = 13.8; 42 day: mdx = 21.6, mdx 0.25% GTE = 21.8, mdx 0.5% GTE = 22.6).

3.3. Systemic indicator of muscle damage

Serum CK activity was not significantly decreased in GTE treated mice at age 28 days (Fig. 3). However, at age 42 days there was a significant decrease (~83–85%) in 0.25% (1345.5 U/L) and 0.5% (1194.3 U/L) GTE treated mdx mice when compared to untreated mdx (7896.5 U/L) ($P \leq 0.05$). There was no significant difference in CK activity for age 28 days when compared to age 42 days. CK activity for GTE treated mdx mice was still considerably elevated when compared to C57BL/6J mice (86.8 U/L).

3.4. Muscle lesions in GTE treated mdx mice

Histopathological features of GTE treated and untreated mdx mice were not significantly different at age 28 days. By age 42 days, 38% of the TA area in untreated mdx mice had normal fiber morphology, while 50% and 48% normal morphology was observed for 0.25% and 0.5% GTE treated mdx mice, respectively (Figs. 4 and 5). This corresponds to a significant improvement of 32% (0.25% GTE) and 26% (0.5% GTE) in normal fiber morphology for GTE treated mdx mice ($P \leq 0.05$). At age 42 days regenerating fibers were the primary histopathological feature that accounted for 61% of the TA area in untreated mdx mice, but only 48% in 0.25%, and 50% in 0.5% GTE treated mdx mice. This is a significant decrease of 21% (0.25% GTE) and 18% (0.5% GTE) in regenerating fibers when compared to untreated mdx mice ($P \leq 0.05$). There were no significant changes in the percent area of immune cell infiltration, necrosis, and degeneration. These markers do not appear to contribute substantially to the increase in normal fiber morphology observed in GTE treated mdx mice.

Macrophage infiltration is a major histopathological feature of the dystrophic disease processes (Fig. 6). Infiltrating macrophages are found throughout mdx TA muscles with dense foci near necrotic, degenerating and regenerating muscle fibers. GTE is thought to reduce inflammation; however, GTE treatment at 0.25 or 0.5% did not significantly reduce macrophage infiltration in mdx mice age 42 days.

3.5. NF-κB in necrotic and regenerating fibers

NF-κB is a transcription factor that regulates the expression of many inflammatory genes. An antibody specific for the phosphorylated p65 (p-p65) subunit of NF-κB was used to determine if GTE had an effect on activated NF-κB immunostaining (p-NF-κB) (Fig. 7). Activated NF-κB is normally sequestered to nuclei; however, in DMD patients necrotic muscle fibers have been reported with cytoplasmic p-NF-κB immunostaining. In TA muscles from mdx mice age 42 days, fibers with cytoplasmic staining were also observed. To confirm that cytoplasmic staining was specific to these fibers, negative control sections were stained without primary antibody. All negative control sections showed minimal background staining, no staining of nuclei, and no fibers with cytoplasmic staining were observed (data not shown). In tissues probed with anti-p-NF-κB, fibers with cytoplasmic staining were typically invaded or surrounded by inflammatory cells that also stained positive for p-NF-κB. The presence of inflammatory cells in and around fibers with cytoplasmic NF-κB staining indicates these muscle fibers were undergoing the process of necrosis. There was no difference in the number of fibers with

Fig. 1. Histopathological features of TA muscles from C57BL/6J and mdx mice age 28 days. (A) Normal muscle morphology was seen in C57BL/6J muscle sections. (B and C) Regenerating fibers (arrow), degenerating fibers (arrowhead), necrotic fibers (asterisk) and immune cell infiltration (double arrow) were apparent in mdx muscle sections. 400× H&E.
cytoplasmic NF-κB staining for GTE treated and untreated mdx TA muscles at age 42 days (∼5 fibers/mm² of muscle tissue). As expected, the number of mdx fibers with cytoplasmic NF-κB staining was elevated when compared to C57BL/6J control (0.1/mm² of muscle tissue). After being phosphorylated, NF-κB enters the nucleus to regulate gene expression. NF-κB has been implicated in muscle fiber regeneration and may contribute to the dystrophic disease process.25 Nuclei in C57BL/6J mice had little or no immunostaining. Both GTE treated and untreated mice had strong immunostaining in peripheral and centralized nuclei. Strong staining in regenerating fibers was observed for 44% of centralized nuclei in untreated mdx, while only 29% and 30% were observed in 0.25% and 0.5% GTE treated mdx, respectively. This was a significant decrease of 34% (0.25% GTE) and 31% (0.5% GTE) for GTE treated mdx mice when compared to untreated mdx mice (P ≤ 0.05).

4. Discussion

The objective of this study was to characterize and identify distinct features of dystrophic muscle pathology during the early disease time course and determine what features of dystrophic muscle pathology were reduced by pre-natal and early dietary intervention with GTE. Recent studies have relied on the percent area of centrally nucleated fibers and the area of necrotic muscle tissue to assess changes in muscle pathology and often combine these features to determine overall affected muscle tissue.7,12,22,26–32 However, these studies have not discriminated the percent area...
of the individual fibers undergoing the histopathologically identifiable processes of degeneration, necrosis or regeneration. Therefore, it is important to note that whole muscle tissue necrosis/degeneration values that are often reported are not the same as the percent area of fiber necrosis or fiber degeneration reported here. One limitation to this approach is that the total area of tissue necrosis may not be fully realized. To compensate for this limitation, areas of immune cell infiltration where there was ongoing tissue necrosis outside of intact fibers were also measured as a separate histopathological marker and quantification of the number of infiltrating macrophages was also reported.

The time course of histopathology shows that the percent area of normal fiber morphology is the same for C57BL/6J and mdx TA muscles at age 14 days, but by age 21 days there is a significant decrease in the percent area of normal fibers for mdx. This time point represents the onset of disease for the mdx mouse. Normal fiber morphology at all other ages analyzed was significantly decreased for the mdx mouse. The percent area of muscle fiber necrosis, degeneration, and immune cell infiltration represent important features of the disease process between ages 21 and 28 days, but overall account for only small percentages of the abnormal fiber morphology (~4.5–6%). The percent area of regenerating fibers represents the largest portion of abnormal fiber morphology at ages 28 (28%) to 42 (61%) days. The large percent area of regenerating fibers compared to other histopathological features indicates that the regenerative process is a substantial contributor to dystrophic muscle pathology. Studies detailing the process of muscle regeneration have shown that in damaged muscle tissue, fibers are capable of returning to normal fiber morphology; however, many regenerated fibers retain centrally located nuclei indefinitely.33–36 The persistence of centrally located nuclei is believed to signify that abnormal processes are occurring in these cells that prevent the nuclei from returning to the periphery of the cell.33–36 From this initial analysis, it was determined that for the GTE treatments muscle pathology should be examined at ages 28 and 42 days because these time points represent the early degenerative and regenerative disease stages.

Serum CK is a systemic indicator of muscle pathology. CK is normally restricted to the cytoplasm of muscle fibers, but when the fibers are damaged it is released into the serum. Serum CK was not different for GTE treated mdx mice compared to untreated mdx at age 28 days, but at age 42 days there was a significant improvement in GTE treated mdx mice. Similarly, there was no difference in

Fig. 5. Quantification of histopathology for mdx and GTE treated mdx TA muscles at ages 28 and 42 days. (A) Although necrotic fibers and fiber degeneration/regeneration were apparent in mdx TA muscles by age 28 days, there was no difference in histopathology in GTE treated mdx mice compared to untreated mdx mice at age 28 days. (B) By age 42 days there was a significant increase in normal fiber morphology and a decrease in regenerating fibers in GTE treated mdx mice. *Mean untreated mdx values were significantly different compared to treated mdx mice (P < 0.05).

Fig. 6. Macrophage infiltration (F4/80) of TA muscles from mice age 42 days. (A) C57BL/6J muscle sections had very few infiltrating macrophages. Untreated mdx sections had macrophage infiltration (arrow) throughout with dense foci of macrophages. Treated mdx sections from 0.25% GTE and 0.5% GTE had similar macrophage infiltration (arrow) compared to untreated mdx. 100 x. (B) Quantification of macrophages revealed there was no difference in the number of infiltrating macrophages for GTE treated and untreated mdx mice.
normal fiber morphology for GTE treated and untreated mdx mice at age 28 days; however at age 42 days there was a significant improvement for GTE treated mdx mice. There was up to a 32% increase in normal fiber morphology for these mice. The improvement in normal fiber morphology was largely accounted for by up to a ~21% decrease in regenerating fibers. Other histopathological features were unchanged by GTE treatments. Collectively this data indicates that overall muscle pathology was decreased in GTE treated mdx mice.

To further assess how GTE treatments modulate inflammation; macrophages and NF-κB immunostaining were quantified. There was no difference in the number of macrophages infiltrating the TA muscles in GTE treated or untreated mdx mice. Although GTE is believed to have anti-inflammatory properties, it does not appear to decrease the number of infiltrating macrophages in mdx mice at age 42 days. However, a recent study reported that muscle from mdx mice contains two subpopulations of macrophages, M1 and M2.8 M1 macrophages are thought to be cytotoxic and pro-inflammatory while M2 macrophages inhibit the cytotoxicity of M1 macrophages and promote muscle regeneration. In mdx mice there was a shift in the macrophage phenotype to the regenerative subset between age 4 weeks and 12 weeks.8 Although there was no change in the overall number of infiltrating macrophages in GTE treated mdx mice age 42 days, one possibility is that GTE induces a shift in the phenotype of infiltrating macrophages. If GTE treatments did contribute to a shift in macrophage phenotype from M1 to M2 without affecting the total numbers of macrophages, it could result in an improved regenerative process. However, further studies are needed to address this possibility.

A component of GTE, EGCG, has been reported to inhibit IKK activity which results in inhibition of NF-κB.10,13–15 Acharyya et al.25 recently reported mdx muscles lacking IKKβ, a subunit of the IKK complex, have an increase in regenerative capacity when compared to control mdx. They also found that mdx muscles with macrophages lacking IKKβ had decreased muscle pathology and a decrease in the expression of pro-inflammatory makers.25 Their data suggest a mechanism through which NF-κB signaling in macrophages and regenerating muscle fibers promotes degeneration and represses regeneration.25 Several studies have reported an increase in the number of regenerating fibers after treatments that reduce or block NF-κB activity while others have reported a decrease in regenerating fibers.25,28,29,37,38 These differing results may stem from the way in which the NF-κB pathway is altered by treatment compounds and the methods used to administer these compounds (i.e. age of treatment initiation and termination, route of administration). Our findings indicate that pre-natal and early dietary GTE treatment reduces the amount of regenerating fibers potentially through alterations in NF-κB signaling. Past studies have also shown a decrease in muscle necrosis when NF-κB activity is inhibited.25,28,29 Our results indicate that the amount of fiber necrosis is unchanged by GTE treatment. Additionally, the number of fibers with cytoplasmic NF-κB immunostaining was unchanged by the GTE treatment. The presence of inflammatory cells infiltrating fibers with cytoplasmic NF-κB staining signifies these muscle fibers were undergoing necrosis. These findings suggest that while muscle degeneration and necrosis initiates the process of muscle wasting, impaired muscle regeneration plays a key role in the progression of muscular dystrophy.

GTE has been shown in several studies to decrease serum CK levels and muscle pathology while increasing force output, and endurance capacity in mdx mice.12,22,30,39 The anti-oxidant properties of GTE are believed to be the primary element responsible for these improvements, other signaling pathways may also be responsible. Previous reports have shown that GTE treatment can result in a significant decrease in muscle pathology,12,22 which is supported by this study. However, past studies did not discriminate the different pathological markers that were reported here, including the finding that the primary change was in the percent area of regenerating fibers. In this study and previously we reported that serum CK was decreased with pre-natal and early GTE...
treatment. Serum CK is an indicator of the extent of muscle damage and a significant decrease of this marker indicates that GTE is having an important impact on dystrophic muscle pathology. Additionally, no other studies have attempted to determine the effect of dietary GTE on inflammation. Although no significant change in infiltrating macrophages was observed, a decrease in NF-κB immunostaining in regenerating fibers was observed. NF-κB is a known regulator of inflammatory genes and may play an important role in the dystrophic disease process and the recruitment of inflammatory cells. GTE role in inflammation may prove to be an important component of the decreased mdx muscle pathology.

CAM approaches, including the use of anti-inflammatory botanicals in general and GTE in particular, may lead to the amelioration of DMD and provide important insight into disease processes. CAM interventions can be used in conjunction with conventional therapies in a cost-effective manner to improve disease prognosis. GTE treatments of mdx mice resulted in increased normal fiber morphology, decreased regenerating fibers, and decreased NF-κB staining in regenerating fibers. The detailed histopathological analysis revealed that changes in the percent area of regenerating fibers, necrotic fibers, and immune cell infiltration were small in comparison to the change in regenerating fibers. This data indicates that GTE decreases NF-κB activity in regenerating fibers which may result in changes in the regenerative process leading to an increase in normal fiber morphology. Detailed muscle histopathology for pre-natal and early GTE treated mdx mice has not been previously reported and reveals that GTE may be an additional treatment option to decrease early muscle pathology for this incurable disease. In conclusion, GTE decreases muscle pathology potentially by suppressing NF-κB activity in regenerating fibers of mdx mice. While GTE did not decrease overall immune cell or macrophage infiltration, additional studies are needed to examine the role of GTE in modulating the shift of macrophages towards M2 phenotype and thereby ameliorate the dystrophic disease process by favoring anti-inflammatory responses.

Conflict of interest

The authors have no conflict of interest.

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References


